

Determining Feulgen-DNA of Individual Chromosomes by Fluorescence Cytophotometry with Incident Light*

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Summary. A microfluorometric method for measuring the DNA content of chromosomes is described. The measurements were made with the MPV (microscope photometer with adjustable measuring diaphragm, E. Leitz, Wetzlar) with an incident light fluorescence illuminator of 0,05 % Feulgen (pararosaniline) stained chromosomes of the cell line B 14 FAF 28 of the Chinese hamster. The variation coefficient of the measurements ranged between 4 and 11 %. The measured values correlate well with DNA measurements quoted in the literature for absorption cytophotometry and length measurements for the same chromosomes.

Introduction

Knowing the DNA content of individual chromosomes is of significance in comparative investigations (chromosomes of different species, corresponding chromosomes in the same species), in identifying chromosomes and determining losses or increases of substance in individual chromosomes. The determination of the DNA content of chromosomes has been shown to be superior to usual morphological methods (determination of chromosome length, position of the centromere, satellites) and auto-radiography (Nitsch and Murken, 1970). The results of chromosomal DNA estimations are more accurate than measurement of length (Rudkin, 1965), they are independent of the state of contraction of the chromosomes and of the spreading of the filaments. Using DNA, a direct reference to the genetically active material in the chromosome is obtained, assuming that within one given chromosome this is constant.

Various methods have been used to calculate the DNA content of chromosomes (Bailly, 1967; Carlson *et al.*, 1963; Gaillard *et al.*, 1968; Mendelsohn *et al.*, 1964; Radley, 1965; Rudkin and Schulz, 1961; Rudkin *et al.*, 1964; review Nitsch and Murken, 1970). Since a fluorometric method requires less expenditure of apparatus and time, this was chosen to determine the DNA content of chromosomes. Fluorometric procedures are free from the disadvantages of the absorption technique. Consideration of the distribution of the material, and then measurement of its surface area is not necessary.

However, with this method, difficulties such as fading of fluorescence, reabsorption, and a partial non-linear relationship between fluorescence intensity and fluorochrome concentration must be considered and eliminated. Auramine 00 (Rozanov and Kudryavtsev, 1967), acridine yellow (Böhm and Sprenger, 1968),

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acriflavine (Böhm and Sprenger, 1968), preparation 33258 from "Hoechst" (Herzog and Schütze, 1968) and pararosaniline (Ploem, 1967) all can be used as DNA specific fluorochromes.

In this paper we describe a quantitative fluorometric method for determining the DNA content of chromosomes using the fluorochrome pararosaniline. Chromosomes of the Chinese hamster ($2n = 22$) were used; our results are compared with those given in the literature for absorption (Carlson *et al.*, 1963), and length measurements (Hsu and Zenzes, 1964).

Materials and Methods

Cell cultures of the male cell line B 14 FAF 28 of the Chinese hamster (HSU *et al.*, 1962) (Fig. 1) were incubated in 0.06 γ - % colchicine for 4 hours, then treated hypotonically with 1 % sodium citrate and fixed in methanol-glacial acetic acid (3:1). The preparations were examined by phase-contrast for good spreading mitoses and then stained in the following way: fixed again in formaldehyde-methanol (1:9), washed in methanol, hydrolysed in 4N HCl for 105 minutes (Böhm, 1968) stained with either 0.05 % or 0.01 % pararosaniline (instead of 0.5 %, after Graumann, 1953), thoroughly washed in bisulfite and tap water, dehydrated in 70 % and 90 % alcohol (2 minutes each), then twice for 5 minutes each in absolute alcohol, washed 2 minutes in absolute alcohol-xylol (1:1), and finally given 3 washes, of 5 minutes each, in xylol. The preparations were mounted in non-fluorescent Cargille oil $n = 1.556$ (Cargille Lab., Cedar Grove, N. Y., USA). To prevent fading the preparations were stored in darkness. Three controls were prepared: one only fixed; one hydrolysed but not stained; and one not hydrolysed but stained.

Measuring Instruments and Measuring Procedures

The measurements were made with the MPV (microscope photometer with variable measuring diaphragm, E. Leitz, Wetzlar) (Thaer, 1966; Weber, 1965). A detailed description of an instrument with transmitting phase-contrast coupled with incident fluorescent excitation (Grehn and Kornmann, 1965) is given by Böhm and Sprenger (1968). Therefore we will only discuss those differences between this (Böhm and Sprenger, 1968) and our system which relates especially to the demands of our measurement problem.

The chromosomes were located by phase-contrast (after Zernike, 1942), using objective Phaco NPL oil 100/1.30. To ensure that the chromosomes were exposed to the least possible light from the tungsten lamp, a 2 mm OG 570 filter (Schott und Gen., Mainz) was inserted in the light path. Instead of using the opakilluminator with a neutral light splitting plate (Grehn and Kornmann, 1965) we used the new fluorescence illuminator (Böhm and Sprenger, 1968; Kraft, 1969; Ploem, 1967a, b; Ploem, 1969), which is fitted with four dichroitic light splitting mirrors. These mirrors reflect about 90 % of the light up to a well-defined or critical wave length and have a high transmission of about 90 %. Light not reflected by the mirror but passing it, is absorbed in the dark space (Figs. 2, 3). Each mirror has actually a built-in cut-off (barrier) filter which transmits light at wave lengths above that of the reflection of the mirror. This construction of the fluorescence illuminator offers in contrast to a customary illuminator an increase in fluorescence intensity by a factor of about 4. Exciting light and emitted light (fluorescence) are precisely separated. This results in the preparation having the dark background so important for microfluorometry.

A lateral light source produces a beam which passes through an external light-field diaphragm before entering the fluorescence illuminator. To keep the

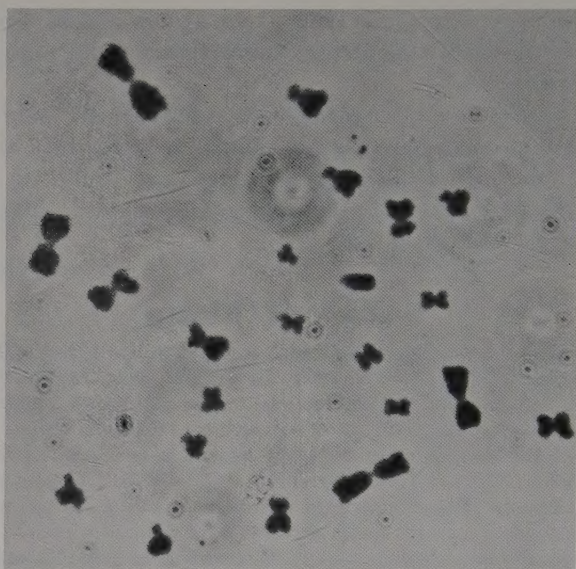


Fig. 1. Chinese hamster: male karyotype of cell line B 14 FAF 28. Y chromosome without short arms

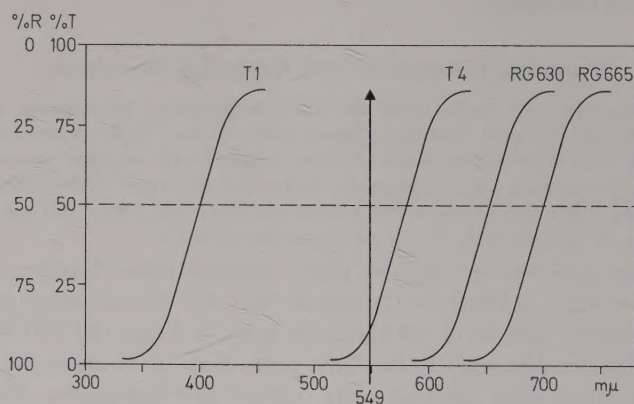


Fig. 2. Plan of the critical wave lengths of the beam splitting mirrors (T_1 , T_4) and the cut-off (barrier) filters (RG 630, RG 665) in the spectrum, shown against reflection (% R) and transmission (% T). The reflecting property of the mirror is shown at wave lengths lower than that of the critical wave length. The light which is above this critical wave length is transmitted. The cut-off filters absorb light up to the critical wave length, the light above that is completely transmitted. The arrow indicates the position of the exciting wave length for pararosani-line in the spectrum

light source as constant as possible, we used the 150 W Xenon lamp with a magnetic constant-voltage control (Wedemeyer, 1964) (Dominitwerke, Brilon). The exciting light was filtered with a 549 nm interference filter (band width 11 nm) together with a 5 mm thick red-suppressing filter BG 38. Beyond the dichroitic splitting mirror with a critical wave length of 580 nm (position 4 on fluorescence

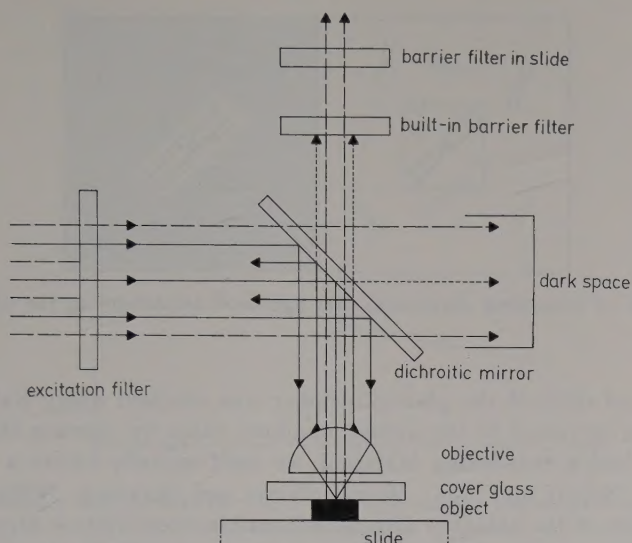


Fig. 3. Diagrammatic representation of the light path in the fluorescence illuminator. Mirror setting 4 with green reflecting interference light splitting mirror. Straight line: exciting light; horizontal heavy- and thin-hyphenated line: absorbed light; vertical thin hyphenated line: light absorbed by the built-in cut-off filter; vertical heavy-hyphenated line: fluorescence light (after Ploem, 1969)

illuminator)¹ the monochromatic beam reaches the Feulgen-stained chromosome and excites it to a red fluorescence. The emitted fluorescent light can pass straight through the dichroitic mirror, passes the K 580 filter behind the mirror and strikes the second filter RG 655 (Schott und Gen., Mainz). Of the total fluorescence the fraction from 580 nm to 665 nm is filtered out, the remaining light passing through to be observed or to go to the photometer (Fig. 3).

Between the interference filter and the fluorescence illuminator there is a shutter, so that the time of excitation of the object can be controlled.

The fluorescence light (Fig. 3) finally reaches the photocathode of the red-sensitive photomultiplier (Type 9558 A, S-20 photocathode, EMI, England), which takes its extraction voltage from the highly stable power pack NSHM BN 600 (Knott-Elektronik, Hohenschäftlarn).

Measurement of the photomultiplier signal is made with a recorder Servogor RE 511 (Metrawatt, Nürnberg), which is specially adapted to the EMI photomultiplier (Pickhardt and Robock, 1965) (through Knott-Elektronik, Hohenschäftlarn).

The screws of the upper part of the photometer, containing the measuring diaphragm, were loosened far enough so that it could be turned, and therefore the chromosomes in every position in the preparation could be accurately framed in the diaphragm for measurement (Fig. 4). In a similar simple way, the light-field diaphragm could also be altered by turning it about the optical axis. To do this, we merely took off the guard.

1 When the chromosomes are examined by phase-contrast, the mirror with the critical wave length of 400 nm (position 1 on fluorescence illuminator) must be used (Figs 2, 3). It gives a picture with better contrast than when mirror 4 is in the light path.

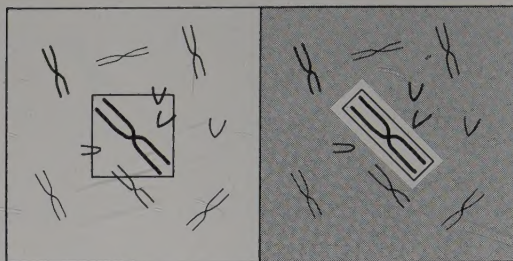


Fig. 4. Position of measuring diaphragm and lightfield diaphragm in the objective plane

The natural drift of the photomultiplier was checked every half hour. The deviation was corrected to the actual standard value by altering the extraction voltage. To find a non-fading standard we used initially either a 2 mm thick GG 21 glass (Schott und Gen., Mainz) (Böhm and Sprenger, 1968) or a 3.42×10^{-5} m solution of the azlacton of p-dimethylamino-benzylidene-hippuric acid in N.N dimethylformamide (Eisenbrand and Hauprich, 1967) ($d=1$ cm), but in later experiments the GG 21 glass was used, because it was simpler to use. Excited with a 5 mm BG 12 filter together with a 0.2 % transmitting grey filter it yields a green fluorescence similar to that of the chromosomes (percent fluorescence, not fluorescence colour) and which is filtered by a K 530 cut-off filter. For standardisation the light field diaphragm (centreable iris diaphragm) was kept fully open (about 30μ in objective plane) and also the measuring diaphragm (centreable iris diaphragm, about 20μ in the objective plane). With these diaphragm settings and the conditions for measurement as given above, a value of 40 % is recorded. In one measuring session for all the chromosomes of one metaphase lasting one to three hours, a maximal variation of ± 3 % is registered.

The size of the measuring diaphragm is first set four the chromosome to be measured, using phase-contrast (Fig. 4). Then a nucleus as far from the mitosis as possible is moved into the field and is made to fluorescence by opening the shutter. This allows the adjustment of the light-field diaphragm to the size of the measuring diaphragm. After closing the shutter (Compur-Werk, München), and using transmitting light the chromosome is brought into the field again, focussed, made to fluorescence, and measured. For small chromosomes the iris diaphragm was used, for the larger ones the rectangular diaphragm.

The light field and measuring diaphragms were made to fit the shape and size of the chromosome, with the light field diaphragm always about 1μ wider open (Fig. 4). The largest measuring diaphragms used for the chromosomes in group I of the Chinese hamster (Hsu and Zenzes, 1964) are $12 \times 4 \mu$. Mitoses were measured only if all the chromosomes were relatively straight and consequently the diaphragms could be adjusted to fit them. With diaphragms of this order of size, the values of the background are always zero, i.e. they cannot be recorded. For the largest opening of the centreable iris measuring diaphragm with a diameter of 20μ the background levels lie between 0 and 3.5 %.

With a $25\times$ ocular chromosomes were observed at a magnification of about $5000\times$ and their surroundings examined for background fluorescence with a

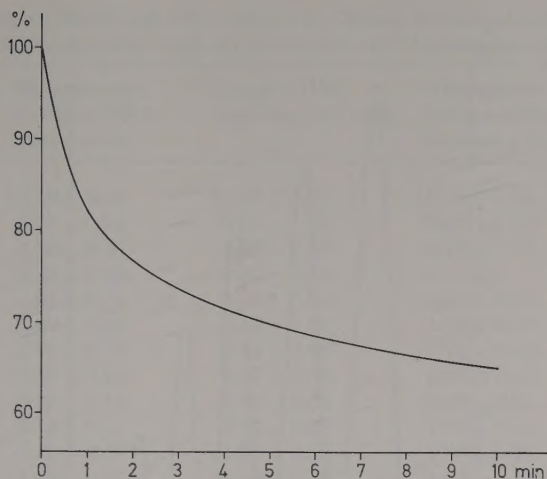


Fig. 5. Fluorescence fading curve of a 0.05 % Feulgen-stained chromosome of group I of the Chinese hamster

rectangular diaphragm of about $1 \times 1 \mu$. When the measuring diaphragm excluded the chromosome completely, no emission was recorded. As the chromosome was slowly moved beneath the objective, the reading climbed to a maximum value at the point where the chromosome filled the diaphragm.

The total "darkness" of the background within the aperture of the diaphragms as used, allows the alteration of their size relative to that of the chromosomes. Nevertheless one and the same chromosome produces a higher fluorescent intensity if the correctly adjusted measuring diaphragm is opened further, with the size of the light field diaphragm unchanged. This was traced back to the effect of stray light in the photometer.

The measured mitoses were exposed to the light of the tungsten lamp for up to 2 hours in the same experiments. To show whether this led to a fading of those chromosomes measured later, either the larger or the smaller chromosomes were measured first alternately. No measurable fading could be established.

The measurements were carried out on the preparations stained with 0.05 % pararosanilin. We also attempted to measure mitoses stained with 0.01 % pararosaniline which led to similar results. The weak contrast, however, produced difficulties in focussing the object, which could be avoided if one did not mount the preparations, but covered them with immersion oil immediately after staining.

Results

The control preparations which were either fixed or hydrolysed only, but not stained, showed no fluorescence when the cut off filter K 610² (Schott & Gen., Mainz) was used, while the non-hydrolysed but stained preparation gave a pale reddish fluorescence which was probably produced through hydrolysis during the staining treatment, because of HCl in the Schiff's reagent.

² This filter had to be used because the RG 665 gave a too dark picture.

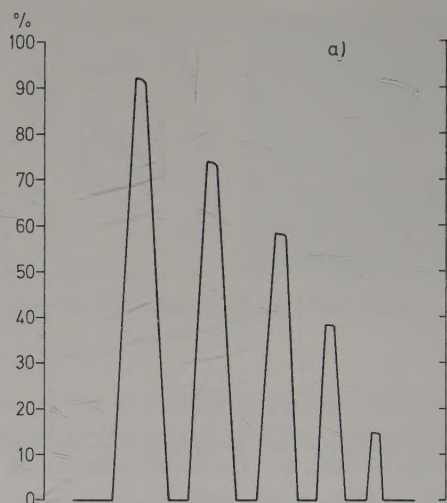


Fig. 6. Measured levels for each of 5 different large chromosomes. Measurements were made with an extraction voltage of 1.1 KV with the recorder sensitivity set at 50 mV and between 5 and 95 % of the breadth of the paper. The recorder needs one second for full registration

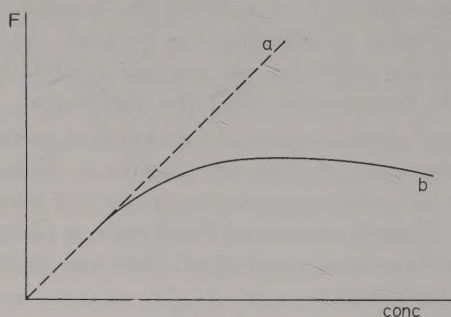


Fig. 7. Fluorescent intensity (F) plotted against fluorochrome concentration (conc). A linear ratio (a) is given only by low concentrations. From a certain level this ratio is distorted (b) (after Perrin, 1924)

Fig. 5 shows the fading curve of the fluorescence intensity of the 0.05 % Feulgen-staining, measured for one chromosome of group I of the Chinese hamster with a K 610 cut-off filter.

In Fig. 6 the fluorescence intensity of each of 5 different chromosomes are shown beside one another. They were drawn with a recorder speed of 600 mm per minute. The maximal level can be ascertained, because the decrease is linear during the time of illumination.

A proportional relationship between the DNA content of the chromosomes and the intensity of fluorescent light must be assumed before applying the method (Perrin, 1924; Ruch, 1966; Ruch and Bosshard, 1963). This proportion is only given by low dye concentration (Fig. 7). Böhm and Sprenger (1968) state that

Table. *Measurements of length and DNA-content of Chinese hamster chromosomes of different authors compared with our fluorescence DNA-measurements*

Chrom. No.	Fluorescence Feulgen-DNA Nitsch <i>et al.</i>	Length HSU and Zenzes (1964)	Absorption Feulgen-DNA Carlson <i>et al.</i> (1963)
1	11.56 ± 0.42	11.07 ± 2.09	10.78 ± 0.64
2	9.02 ± 0.44	8.08 ± 1.70	9.07 ± 0.93
X	5.36 ± 0.39	4.78 ± 0.67	5.12 ± 0.74
Y	2.92 ± 0.32	4.25 ± 0.75	—
4	5.93 ± 0.38	5.06 ± 0.83	5.57 ± 0.44
5	3.80 ± 0.32	4.46 ± 1.00	4.33 ± 0.62
6	4.03 ± 0.23	3.84 ± 0.68	3.71 ± 0.52
7	3.59 ± 0.26	3.26 ± 0.59	3.37 ± 0.48
8	2.82 ± 0.16	3.26 ± 0.59	3.06 ± 0.24
9	2.14 ± 0.21	2.36 ± 0.36	1.97 ± 0.22
10	1.89 ± 0.18	1.93 ± 0.32	1.66 ± 0.26
11	1.23 ± 0.07	1.93 ± 0.32	1.27 ± 0.17

The mean value and standard deviation for each chromosome pair and for X and Y chromosomes are given, expressed as a percentage of the total chromosome set. The fluorescence levels represent the average of 26 measurements for each chromosome because 13 metaphases were measured. Chromosome classification after HSU and Zenzes (1964).

a linear relationship between fluorescence and concentration exists only up to an absorbance of 0.2 units. Using the UMSP I (Zeiss, Oberkochen) (Trapp, 1966) we determined the absorbance value of 0.05 % Feulgen-stained chromosomal DNA to be less than 0.1, and therefore it lies within the linear region. For the 0.01 % staining the values are even lower.

In the Table the fluorescence levels (relative DNA content) of the individual chromosome pairs of the Chinese hamster are shown; the figures obtained by Carlson *et al.* (1963) by an absorption method for the female chromosome set, and those of Hsu and Zenzes (1964) for length measurements of male chromosomes are given for comparison. They show good correlation. The relatively low level for the Y-chromosome is caused by the lack of the short filaments of this chromosome (Fig. 1). The variation coefficient of the fluorometric DNA measurements is between 4 and 11 %, that of absorption measurement 6—16 % and of length measurement 14—22 %. An inverse proportionality between DNA content and variability is not indicated by our results.

Discussion

Quantitative fluorometric estimation of nucleic acids with basic dyes was tried earlier by Bosshard (1964), Rigler (1966), and also by Böhm and Sprenger (1968). Böhm and Sprenger (1968) in their experiments showed good correlation between the DNA content of guinea pig sperms, mouse lymphocytes, granulocytes, and liver cell nuclei as measured fluorometrically and that of absorption cytophotometry of the same objects, using acridine yellow, acriflavine, coriphosphine 0, and pararosaniline as fluorochromes.

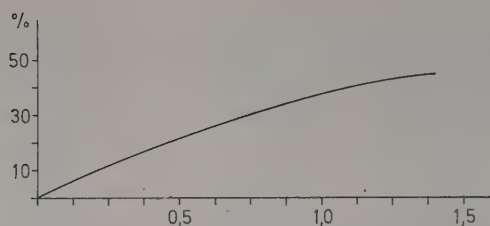


Fig. 8. Diagrammatic representation of the error in dependence of the absorbance of the stained object (from Rozanov and Kudryavtsev, 1967)

These results show that fluorometric DNA measurement gives reliable and reproducible results. Therefore, it seemed to us to be a method well suited to the investigation of single chromosomes. The values we obtained fluorometrically show good correlation with absorption measurement (Carlson *et al.*, 1963) and length determination (Hsu and Zenzes, 1964) and show at 4–11 % a better variation coefficient than the other measurements (6–16 % and 14–22 % respectively).

Reabsorption phenomena (Böhm and Sprenger, 1968) ("autoabsorption" after Zanotti, 1964) play no part in our experiments because the RG 665 filter (Schott und Gen., Mainz) which we used, is above the range of the absorption spectrum of pararosaniline.

Rozanov and Kudryavtsev (1967) assume on the basis of the formula

$$\frac{\Delta Q}{Q} = - \left(1 - \frac{1 - e^{-A}}{A} \right) \times 100 \%$$

(where Q is the particular material and A is the optical density) and the resulting graph (Fig. 8), that it is possible to calculate the standard error of fluorometric measurements which is dependent upon the optical density. In Böhm and Sprenger's (1968) experiments one would expect an inaccuracy of about 18 %, whereas in our own ones of about 4 %. Nevertheless the fluorometric results of Böhm and Sprenger correlate well with those of absorption cytophotometry and there is no basis for an error of 18 %.

Cytophotometric investigations of chromosomes are beset by difficulties and errors in preparation:

1. The preparing of good spreading metaphases and the removal of cytoplasmic residues to give a background as blank as possible;

2. the loss of DNA during fixation (methanol-glacial acetic acid) (Gaillard *et al.*, 1968) through the action of the acetic acid, hypotonic treatment (Rudkin *et al.*, 1964) and hydrolysis by the Feulgen-stain (Gaillard *et al.*, 1968).

Gaillard *et al.* (1968) describe a sedimentation technique in which the chromosomes were fixed with a formaldehyde-methanol mixture (1:9) instead of methanol-glacial acetic acid. They obtained higher absolute DNA levels with this method than after methanol-glacial acetic acid fixation. Nevertheless, we ourselves did not succeed in applying the method following their instructions.

Rudkin *et al.* (1964) concludes from a lower value for A_T (total absorption of a chromosome) for chromosomes treated under especially strong hypotonic conditions, that this DNA loss is a result of this treatment. Contrary to this, Garcia (1968) found a 7—12 % increase in the DNA content of rabbit leucocytes after treatment with different hypotonic media. Through squash preparations such as those Bailly (1967) and Carlson *et al.* (1963) used, the hypotonic treatment can be avoided. However this method has other disadvantages for cytophotometric measurements, above all poor spreading of the chromosomes.

Gaillard *et al.* (1968) state that the possibility of a 10—20 % loss of DNA through Feulgen-hydrolysis can not be excluded. The Feulgen- and UV-absorption measurements of Bailly (1967) give no reference to DNA loss because of Feulgen-hydrolysis, but they do not exclude it.

After standardising the conditions for preparation, fixation, hydrolysis and staining, the Feulgen reaction is very reliable quantitatively (Sandritter *et al.*, 1966). In our results we quote relative values which are more reliable, if DNA losses relate proportionally to the different chromosomes of a metaphase. Absolute values can be obtained with our method using a reference system [nucleus or chromosome with a known DNA content, or a film of DNA (van Duijn and Persijn, 1960)].

Until now only a few reports of quantitative cytochemical DNA estimations of chromosomes have been submitted. Rudkin and Schulz (1961) determined densitometrically the relative DNA content of individual chromosomes of *Drosophila melanogaster* with a variation coefficient of 5—10 % (by reading a negative, taken in UV-light, with a densitometer).

Carlson *et al.* (1963) published the first DNA estimation for mammalian chromosomes; they determined the DNA content of the 22 chromosomes of the Chinese hamster using direct microspectrophotometry after Feulgen-staining. The variation coefficient as estimated came to 6—16 %. (These values were calculated from the quoted standard variations.)

Rudkin *et al.* (1964, 1965) determined the DNA content of the chromosomes of 5 human metaphases densitometrically. The variability for the complete karyotype lays between 6 and 18 % whereas that for the easily identified chromosomes 1, 2, 3 and Philadelphia were less than 10 %, and for the centromere position (proportion of the length of the larger filament to that one of the whole chromosome) was only half as great as for the whole chromosome.

Radley (1965) reported the absorption cytophotometric DNA determination of 5 metaphases of the black-tailed wallaby *Protemnodon bicolor*. We calculated a variation coefficient of 3—9 % from the values given.

Mendelsohn *et al.* (1964) used a so called CYDAC system a flying-spot microscope connected to a computer. As Feulgen-staining resulted in poor contrast, the chromosomes of 3 human metaphases were stained with gallocyanin-chromalaun after ribonuclease treatment. The variation coefficient was between 5 and 15 %, for the position of the centromere 3—10 %.

The investigations of the DNA content of the 8 chromosome pairs of the salamander *Pleurodeles waltlii* Michah by Bailly (1967) of each of 15 metaphases using Feulgen-absorption and UV-absorption measurements indicate greater

accuracy for the UV measurements. We calculated the variation coefficients to be 2—6 % for UV-light and 3—8 % for the visible spectrum.

For measuring the DNA content of human chromosomes Gaillard *et al.* (1968) used a photographic colorimetric method, described by den Tonkelaar and van Duijn (1964). They established that methanol-glacial acetic acid fixation leads to loss of DNA, which by their sedimentation technique was very much reduced (Gaillard *et al.*, 1968). The reproducibility of their measurements at 2—6 % is very good; the variation coefficient is close to 9 %. There is an inverse relationship between DNA content and variation coefficient. With comparable DNA- and length measurements, the DNA measurement is better than that of length.

From the results of the various authors a range in variation coefficients for DNA measurements for individual chromosomes of 2—18 % exists.

The method described here for microfluorometric DNA determination of chromosomes has the advantage of small outlay for apparatus and little expenditure of time. With a variation coefficient of 4—11 % the measurements have good reproducibility.

At the moment we are measuring the Y-chromosomes of the human karyotype 47, XYY comparatively; the results will be published later.

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L e b e n s l a u f

Am 5. März 1942 wurde ich in Lauenburg (Pommern) als zweites Kind des Dr. med. Barnim Nitsch, Facharzt für Hals-, Nasen-, Ohrenkrankheiten und seiner Ehefrau Hildegard, geb. Zibelius, geboren.

Im Januar 1945 mußte ich mit meinen Eltern flüchten. In Bevensen (Niedersachsen) und später in Siegen (Westfalen) ging ich zur Volksschule. Anschließend besuchte ich sechs Jahre lang den humanistischen Zweig des Städtischen Gymnasiums zu Siegen. Nach dem Umzug meiner Eltern nach Haltern (Westfalen) ging ich bis zum Abitur im März 1963 auf das Gymnasium Petrinum zu Dorsten (Westfalen).

Nach Absolvierung eines "studium generale" am Leibniz-Kolleg der Universität Tübingen begann ich 1964 mit dem Studium der Humanmedizin an der gleichen Universität und legte dort auch das Vorphysikum und Physikum ab.

Nach klinischen Semestern in Tübingen und Edinburgh (Schottland) studierte ich seit Herbst 1968 in München, wo ich am 15.6.1970 das medizinische Staatsexamen bestand.

Seit dem 1.9.1970 bin ich als Medizinalassistent an der chirurgischen Klinik der Universität München tätig.

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For measuring the DNA content of the cells, the following method was used: The cells were fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μ m. The sections were stained with hematoxylin and eosin (H&E) and mounted in cedar oil. The DNA content was determined by measuring the optical density of the stained sections at 540 m μ using a spectrophotometer.

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